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Encapsulation Processes by Bilayer Vesicles

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16.1 Introduction

16.1.1 Vesicular Aggregates

Surfactants, or amphiphiles, comprise a large class of compounds, characterized by one (or more) ionic or highly polar headgroups and one, two, or rarely more, hydrophobic alkyl tails (usually *n*-C_8 to *n*-C_{22}, the most common being C_{12}, C_{14}, C_{16}, C_{18}, and C_{20}). In aqueous media, above a critical concentration, these systems cope with their dual structural properties by forming aggregates in which the headgroups are in contact with water while the alkyl chains reside in the core of the aggregate, avoiding interactions with the aqueous medium.

A variety of different morphologies are possible for these aggregates, depending on the molecular structure of the single amphiphilic molecule, including their size and shape. But other factors like ionic strength, temperature, pressure and pH also play a role.[1]

Vesicles (from the Latin vesicula, small bubble) belong to the most extensively studied amphiphilic aggregates.[2] Not only because the bilayer membrane is the building block of cell membranes, but also because vesicles or liposomes offer the unique possibility for solutes to bind to the outer- and inner leaflets of the bilayer whereas the hydrophobic interior of the bilayer can be used to entrap hydrophobic solutes and also membrane proteins.

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The Gibbs energy of a vesicle depends on a compromise of attractive and repulsive interactions. The main contributions come from hydrophobic interactions between the tails, the major driving force for aggregation, and from repulsive interactions between the headgroups at the aqueous interface, which, in case of ionic surfactants, are in part compensated by counterion binding. This counterion binding is usually stronger for vesicles than for micelles.[3] Other factors also play a role, one of them being the loss of conformational entropy of the hydrocarbon tails in the vesicular core.

Water is the essential medium for surfactant aggregation, although association can occasionally also occur in polar solvents with a high cohesive energy density.[4]

Which factors determine the packing efficiency of the amphiphiles in the aggregate? The dimensionless shape factor or packing parameter (P) provides a relation between the molecular shape of the amphiphile and the preferred morphology of the aggregate in dilute aqueous solution at low ionic strength and at ambient temperatures:[5]

$$P = V/a_0 \times l_c$$

Herein, V is the volume of the hydrophobic chain, a_0 the optimal cross-sectional surface area of the headgroup, and l_c the length of the all-*trans* alkyl tails. It will be clear that the curvature of the hydrophilic/hydrophobic interface is related to the value of P. The relation between the aggregate morphology and P is given in Figure 16.1. The packing parameter concept provides a reasonable and useful rationalization of the rich polymorphism of surfactant aggregates, but it has also been criticized. A significant issue is, of course, that the values of a_0 and l_c are not independent of each other.[6] Nevertheless, extensive studies of a large series of amphiphiles in which the structure was gradually modified showed that the approach worked satisfactorily.[7,8]

It was Bangham who, in the sixties of the past century discovered that liposomes could be made from egg yolk.[9] But phospholipids are by no means unique in this behaviour. Kunitake[10] showed that bilayer vesicles can be obtained from synthetic surfactants in case $0.5 < P < 1.0$, while for $P = 1.0$ a flat bilayer is formed.[11] Bilayer morphologies, usually preferred by double-tailed amphiphiles, are shown in Figure 16.2 and are called lamellar phases. The bilayer consists of two opposing layers of amphiphiles, with the aligned tails facing each other and the headgroups situated at the aqueous interface. In case the two ends of the bilayer close to form a global structure with an aqueous inner compartment, the aggregate is called a vesicle, or a liposome if the amphiphilic constituent is a phospholipid molecule. Mammalian cell membranes may contain microdomains ('rafts') which can also be formed in multicomponent liposomes.[12,13] For the structures of the most common phospholipids in cell membranes, the reader is referred to appropriate biochemistry textbooks.

Vesicles and particularly liposomes are highly useful, although severely simplified, models for biological membranes.

Conventional molecular dynamic (MD) simulations of the formation of small dipalmitoylphosphatidylcholine (DPPC) liposomes in atomistic detail (1017 DPPC molecules in 106.563 waters) have provided mechanistic insights into some general features of liposome formation.[14] In the very rapid initial stages of the aggregation process micellar-like structures are formed. Lipid bridging between these micelles leads to larger, lamellar morphologies, which are, in fact, curved bilayers. Spontaneous development of curvature, resulting from minimization of edge energy, is then claimed to induce formation of

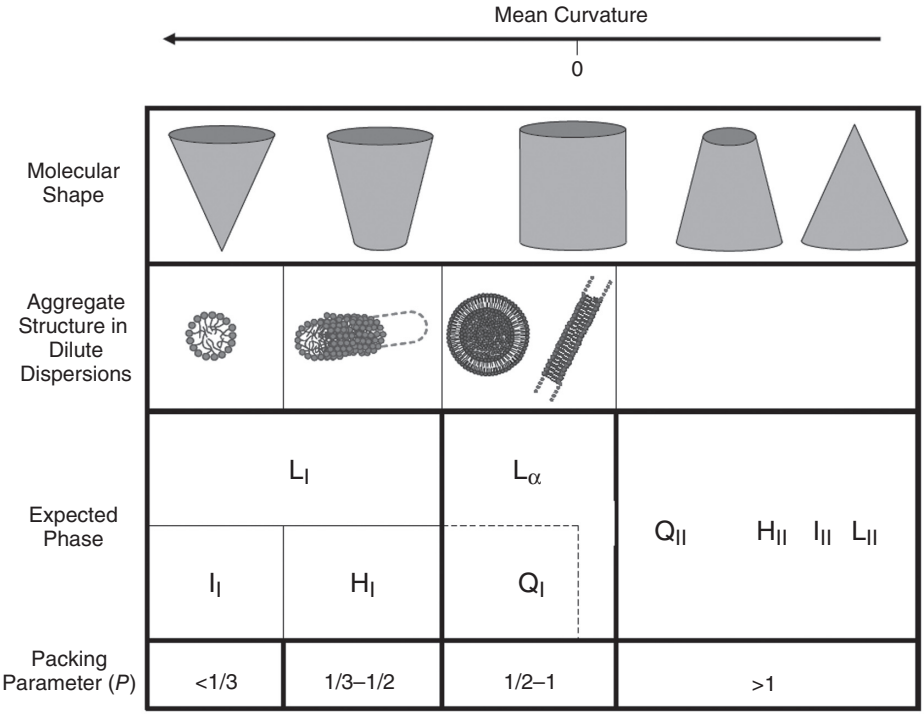

Figure 16.1 *Relationship between molecular shape, aggregate structure in dilute dispersions, phase behavior and packing parameter. Micellar phase (L_1), cubic micellar phase (I), hexagonal phase (H), bicontinuous cubic phase (Q), L_α lamellar phase. Subscripts I and II indicate normal and inverted phases, respectively. From: M. Scarzello, Aggregation Properties of Amphiphilic DNA-Carriers for Gene Delivery, Ph. D. Thesis University of Groningen, p 6, 2006*

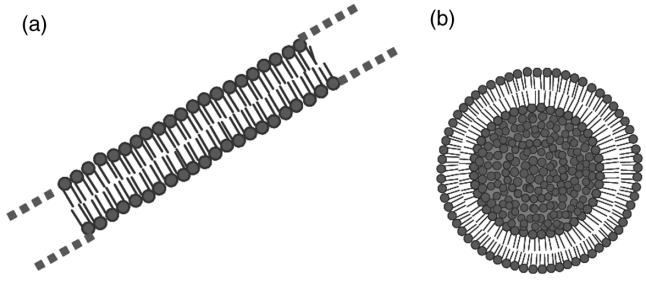

Figure 16.2 *Schematic representation of two possible morphologies of L_α: flat bilayer (a) and a vesicle (b). From: M. Scarzello, Aggregation Properties of Amphiphilic DNA-Carriers for Gene Delivery, Ph. D. Thesis, University of Groningen, p 8, 2006*

liposome-like shapes. These contain water pores with diameters of 1–4 nm, allowing relatively slow equilibration of lipid molecules between the inner and outer leaflets by diffusion. This so-called 'flip-flop' has also been studied in mature vesicles already many years ago.[15] Finally these processes result in collapse of the water pores and formation of the most stable liposomal aggregate.[16,17] Spontaneous formation of vesicles from mixed amphiphiles as well as the interaction of vesicles with other molecules (including cholesterol[18]) has also been investigated using MD techniques.[19]

With a few exceptions (catanionic vesicles, for example[20,21]), vesicles are metastable aggregates and posses a tendency to undergo aggregation followed by merging of the bilayers and eventually precipitation. Therefore vesicles can be best coined as 'colloidal structures'.[22] Apart from catanionic vesicles and contrary to the suggestion by some MD simulations,[14] closed vesicle formation from single amphiphilic molecules is not spontaneous but requires input of mechanical energy such as shaking, stirring, extrusion or sonication, inducing formation of closed vesicles from bilayer sheets and increasing the bilayer curvature and reducing the vesicular size. In fact, the method of vesicle preparation has an effect on the exact lamellar structure and on the vesicular size.[23,24] A bending energy can be defined for formation of a closed vesicle from a bilayer sheet. The curvature stress energy for a bilayer can be calculated with the (simplified) Helfrich Gibbs energy equation.[25] In contrast to micelles, vesicles tend to separate from their dispersions, but this process may take a considerable time span (ranging from seconds to months).

To prove the formation of vesicles a number of indirect techniques can be used such as dynamic light scattering, the use of fluorescent probes and pulsed field gradient NMR self-diffusion measurements. Some more direct techniques such as freeze-fracture and negative staining electron microscopy are less biased by the interpretation of the scientist, but also these methods have their limitations. Cryo-electron microscopy, as introduced by Dubochet in the 80s, [26] is the method of choice when it comes to visualization of small colloidal structures.[27] Recent developments in the vitrification of specimens make it now possible to observe vesicles and other aggregated structures artifact free.

Vesicles with sizes varying from nanometers to micrometers usually contain large numbers of surfactant molecules. Large unilamellar vesicles (LUVs) possess diameters which may vary considerably (50–500 nm) and have a large encapsulation efficiency (5–20 l mol^{-1}). Small unilamellar vesicles (SUVs) with diameters of 20–50 nm encapsulate smaller volumes (0.5–1.0 l mol^{-1}) Under special conditions, giant vesicles may be formed (diameter in the order of 1–500 μm) with a low bilayer curvature, akin to that of a flat bilayer.[28,29] These giant vesicles are able to eject smaller vesicles by a budding process.[30]

In vesicles, the fluidity of the bilayer[31] and the associated stiffness and order of the alkyl tails can vary significantly as a function of temperature, but, of course, also depending on the detailed structure (straight or branched), size, and the presence of one or more double or triple carbon–carbon bonds in the tails. At lower temperatures, the bilayers reside in the gel phase (L_β) with the strongest packing of the all-*trans* alkyl chains and with the largest order and rigidity. Upon increasing the temperature, a cooperative phase transition occurs to the L_α or liquid crystalline phase with a more liquid-like core, allowing fast diffusion of the amphiphilic components, and in which gauche conformations of the alkyl tails are allowed. As expected this main phase transition temperature (T_c) becomes lower if the alkyl chains are shortened or if unsaturation is introduced into the tails.

Sometimes an intermediate 'rippled' phase ($P_{\beta'}$)[32] is first formed from the L_β phase, which transforms to the L_α phase at higher temperatures. At still more elevated temperatures, the lamellar phase may change into inverted cubic (I_β) or related isotropic phases.[33,34] These highly complex aggregates have been proposed as intermediates during membrane fusion. A further temperature increase may lead to formation of inverted hexagonal phases (H_{II}), in which the weakly hydrated amphiphiles are packed in inverted micellar-like cylinders, with the headgroups facing inside and in contact with water in the enclosed compartment.

Vesicles possess many interesting potentials for applications, one of the most important being encapsulation of solutes, a phenomenon that has been investigated in detail and has been utilized in different ways. To get insight into the possibilities for encapsulation, unavoidably involving disturbances of the bilayer structuring and to predict which binding sites are available for solubilizates of different chemical composition, we can have a look at the lateral pressure profile, extensively investigated by Cantor.[35,36] The lateral pressure (LP) is defined as $d\pi(z)$ acting within a thin slice of the bilayer with thickness dz. Positive values of the LP indicate repulsion, negative values stand for attraction. The lateral pressure density is then given by $p(z) = d\pi/dz$, with dimensions of bulk pressures. At the aqueous interface of the membrane, the headgroups repel each other (positive LP), whereas at the interface somewhat deeper into the bilayer, the tails attract each other through hydrophobic interactions leading to a large, negative LP. Still deeper in the membrane, there is little or no water and the enforced partial ordening of the tails is accompanied by a considerable loss of entropy due to their reduced conformational freedom. This means repulsion and a positive LP in the central part of the core of the bilayer. The variation of the LP with the depth in the membrane is an important issue and has been applied in detail in the field of bilayer-protein interactions.[37]

Figure 16.3 shows a LP profile for one mono-layer, the profile for the opposite monolayer is the same. We see how the LP varies with depth in the membrane. Since the formation of a vesicular membrane is associated with a favorable Gibbs energy, the total lateral pressure in the membrane is zero (or nearly so).

The lateral pressure concept provides useful insight into the spatial distribution of forces and is a sum over terms corresponding to layers of finite thickness (dz).[38] Quantitative measurements of the LP remain difficult and even the concept has been criticized.[39]

Chain order parameters, measured by ^{2}H-NMR studies on amphiphiles with specifically deuterium-labelled methylene units in the tails,[40–42] gave a similar view of the distribution of the chain attractions/repulsions as a function of position in the tails. Increasing quadrupole splittings, indicated by an increasing distance between the two peaks in the ^{2}H-NMR spectrum, are associated with increasing chain order.

In sum, a larger repulsion between specific parts of the alkyl chains in a bilayer is reflected in a higher lateral pressure, a decrease in chain order and more area available for that part of the tails. Such changes can be induced by, for example, a higher temperature or introduction of a *cis* C=C bond into the tail or via *trans–cis* isomerization of double-tailed, azobenzene-substituted amphihiles.[43]

In recent years, several computational methods have been developed to calculate LP profiles in membranes. Among them, molecular dynamics (MD) simulations have been particularly successful. Timescales extending to hundreds of nanoseconds are now computationally feasible, but the simulations are still restricted to relatively small system

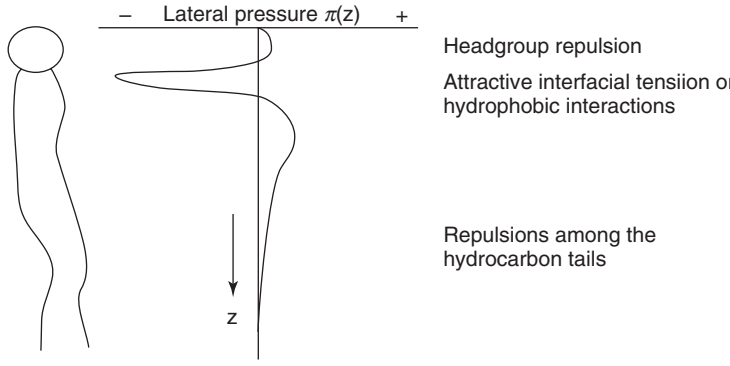

Figure 16.3 *Schematic representation of a lateral pressure profile in a vesicular bilayer. Only one monolayer is shown here, the same profile applies for the other monolayer. The lateral pressure π depends on the depth (Z) in the monolayer. From: M. Kuiper, Azobenzene-substituted Phosphate Amphiphiles : Effect of Light-induced* trans–cis *Isomerisation on Vesicular Properties and the Channel Protein MscL, Ph. D. Thesis, University of Groningen, p 12, 2005*

sizes. MD is also useful in studies of undulatory and thickness fluctuation modes of bilayers.[44]

Finally we note that, apart from double-tailed surfactant molecules, vesicular aggregates can also be formed from a number of other building blocks, including nonionic amphiphiles to form niosomes,[45] single-tailed surfactants[46,47] and complex (co)polymers, polypeptides, and dendrimers.[48] In this chapter we restrict our discussion to encapsulation processes by vesicles formed from synthetic surfactants and phospholipids.

16.1.2 Solute Encapsulation by Vesicles

It will be obvious that the encapsulation of a solute molecule into a vesicular membrane will affect the interchain interactions in the bilayer.[49] Overall, the Gibbs energy for binding should be negative, otherwise the solute would remain in bulk solution. Therefore, partitioning of a relatively apolar solute into the membrane will be most favorable at a location with the best balance between solubilizate-tail and solubilizate-headgroup interactions on one hand and changes in tail-tail interactions on the other hand. These counteracting contributions will also significantly depend on hydration changes. On the other hand, the (solvation) properties of the solubilizate are also different from those in dilute aqueous solution as a result of binding in the vesicular bilayer.

In this brief review we will describe some important encapsulation processes by vesicular aggregates. Encapsulation will be broadly interpreted. Apart from solubilization in the aqueous pool inside the vesicle, the term will also encompass binding of solubilizates to all binding sites available in the vesicular system. We will not discuss bilayer vesicles formed from amphiphiles further functionalized by receptor molecules, such as amphiphilic cyclodextrins.[50]

Instead of a comprehensive review of the vast literature on solute encapsulation, a selection will be made of various illustrative binding possibilities in or at the bilayer.

Although binding to vesicles has been less extensively studied than binding to micelles, attempts will be made to rationalize why specific solute molecules bind to particular binding locations.

16.1.3 Binding Locations

Let us now look at the different locations for spontaneous encapsulation of solutes of different chemical composition. First of all, it goes without saying that encapsulation experiments should be carried out under well-defined conditions. Often vesicles, after their preparation, and subsequently after binding of the solubilizate, need time to arrive at their most stable configurations (size, tail ordering) and adequate attention should be given to this issue. This process may sometimes take days at room temperature. Also the method used for their preparation may exert a definite effect on the vesicular properties. Thus, for a honest comparison of encapsulation abilities, it is recommended that the vesicles are prepared under exactly the same conditions and that their properties are checked by a proper physical technique, such as cryo-electron microscopy. Also temperature control needs attention since the gel and liquid-crystalline phase exhibit different binding efficiencies of solutes.

The binding locations can be charaterized as follows:

(a) In the outer Stern region, where the solute interacts almost exclusively with the polar or ionic headgroups. In case of ionic headgroups, relatively hydrophilic solutes, carrying opposite charges (preferably double-charged) are the best candidates for strong binding. Interaction is mainly governed by electrostatic forces. Close approach between oppositely charged ionic moieties is necessarily accompanied by partial dehydration of the ions, an effect that may in part counteract the attractive interactions.

Hydrophilic solutes like carbohydrates, i.e. glucose, sucrose, and trehalose also bind close to the charged headgroups, replacing hydration water, as laid down in the 'water-replacement hypothesis'.[51] They act as cryoprotectants and help to stabilize the bilayer in case of insufficient water.

There is now compelling evidence that selective hydrogen-bonding interactions between functional groups of hydrophobically-buried molecules residing near the vesicle-water interface are able to overcome the otherwise dominant hydrogen bonding to water molecules, present in high excess. A recent example is the self-adhesion among phospholipid vesicles carrying adhesive agents.[52] Such associations in the aqueous interface are largely governed by entropic factors, as discussed in a beautiful review.[53] The entropic costs for association of ligands tethered to the surface of vesicles are smaller than those for interaction of similar species diffusing independently in three dimensions in an aqueous medium.

A highly unexpected result, first reported in 2003, was the efficient adsorption of hydroxide ions at pH values slightly above 7.5 to (almost) uncharged hydrophobic surfaces[54] and vesicular surfaces formed by reduced-sugar based gemini surfactants.[55,56] The origin of this phenomenon has been investigated in some detail.[57]

(b) In the 'inner' Stern layer, that is at binding locations with negative LP values, at a few methylene units from the headgroups. These are the most frequently encountered binding sites where relatively hydrophobic solutes preferably interact with the partly

hydrated alkyl tails through hydrophobic interactions. Often these solutes also possess a hydrophilic substituent, necessary for sufficient water solubility, and this substituent will be preferentially directed towards the more aqueous region of a bilayer for beneficial contact with water. Incorporation of solutes may disturb the tail–tail interactions to some extent. An example is provided by the anchoring of hydrophobically modified poly(sodium acrylate)s into di-*n*-dodecylphosphate (DDP) vesicles. In case the polymer contains *n*-dodecyl chains, the bilayer is hardly affected as indicated by detailed DSC measurements. By contrast, for *n*-nonyl and *n*-octadecyl chains the hydrophobic mismatch leads to lower main phase transition temperatures and reduced enthalpies of transition per mole of DDP monomer. Another notable consequence of binding of the hydrophobically modified polymer into the vesicle bilayer is the increase of the cooperativity of the melting process, probably resulting from the presence of larger 'patches' with a lower curvature.[58] Another interesting effect induced by hydrophobic mismatching is the clustering of membrane proteins in case their hydrophobic transmembrane domains do not match with the surrounding lipid bilayer.[59]

Interestingly, transvesicular reactions between substrates bound in the exovesicular and endovesicular leaflets have been studied, particularly at relatively low vesicular fluidities.[60]

(c) Deep in the interior of the bilayer. These are the preferred binding locations for strongly hydrophobic molecules. The chain order is relatively low in this region and the LP is positive. There is also more free volume available for incoming hydrophobic solutes. These binding processes will affect bilayer dynamics.

It has recently been shown that introduction of a small, hydrophobic molecule like isoprene may lead to a more ordered and better packed lipid membrane as revealed by MD simulations. The stabilized membrane is then protected against temperature-induced disordering of the tails.[61] Similar thermoprotective effects are induced by cholesterol which also increases the molecular packing of the tails and, in contrast to isoprene, affects the dynamics of the lipids in the bilayer.[62]

(d) In the aqueous inner compartment of the vesicle. Sufficiently hydrophilic solutes can be entrapped in the aqueous medium inside the vesicular system and may favourably interact with the headgroups stacking into this medium.[63] In contrast to the protocols used for binding at the sides labelled (a)–(c), binding in the water pool is realized by preparation of the vesicle dispersion in a medium already containing the solute. During the formation of the vesicular aggregate, the solute becomes entrapped in the inner aqueous compartment. Of course, a prerequisite for this type of encapsulation is that leakage over the bilayer is slow. Carboxyfluorescein, a highly water-soluble fluorescent dye, has been extensively used to probe leakage over vesicular bilayers.[63] Strong bilayer packing, as induced by encapsulated cholesterol, slows down leakage processes.

Finally, we stress that the bound solutes are often certainly not localized at a single, precisely defined, position but may exchange rapidly between various positions of similar binding characteristics and partition coefficients.

As we will discuss below, microencapsulation offers interesting possibilities for reactivity control.[64]

16.1.4 Experimental Techniques for Measuring Encapsulation Processes

It is obvious that binding of a solute to a vesicle will induce changes in the physical properties of both solute and the amphiphilic molecules that make up the bilayer aggregate. Two sorts of information can be obtained from these changes. First, the strength of the interaction, which can be quantified by the solvent/bilayer partition coefficient of the solute. Second, in many cases information can be obtained regarding the location of the binding site although the exact interpretation of the experimental data can be quite challenging. Both types of data are of immediate importance for applications of the particular vesicular aggregates.

A brief summary will now follow of some of the most frequently employed experimental techniques. Several of these have been particularly applied for micellar aggregates, but their usage can often be easily extended to vesicles.

16.1.4.1 Microcalorimetry

The greatly improved sensitivity of the modern microcalorimeters for both differential scanning microcalorimetry (DSC) and isothermal titration microcalorimetry (ITC) has made their application highly useful. Titration microcalorimetry yields binding constants and, when applied as a function of temperature, full thermodynamic details for the binding process.[65] DSC provides details about the disturbance of the bilayer system by the solubilized guest from which conclusions may be drawn about the preferred binding locations.

16.1.4.2 NMR Spectroscopy

Ring current effects have already been examined more than twenty years ago and indicated which groups are in close proximity to an aromatic ring in the solute or in the amphiphile. This is useful information but the exact positioning of these interaction sites within the bilayer may remain a problem.[66,67] Nuclear Overhauser effects have also been employed frequently.

Paramagnetic relaxation enhancement experiments measure the distribution coefficients of solubilizates.[68,69] A comparison is made between the proton spin–lattice relaxation rates of the solubilizate in the absence and presence of noncomplexing paramagnetic ions of the same sign of charge as the headgoups. Such paramagnetic ions reside necessarily in the bulk solvent and their effect on the nuclei of the solubilizate depend on the efficiency of penetration of the solubilizate into the core of the bilayer. The method is applicable for a variety of NMR-active nuclei in the guest molecule. These results can be compared with those obtained from Fourier transform pulsed gradient spin-echo NMR self-diffusion measurements.

16.1.4.3 Phosphorescence, Fluorescence and Steady-state Absorption Techniques

These techniques can be employed for quantitative studies of the distribution constants of solubilizates as well as for measuring the kinetics of solubilization. Binding at the surface or in the core of the aggregate can be distinguished under favorable conditions by fluorescence probing.[70–72]

Solvatochromic probes like Nile Red allow characterization of temperature-dependent phase transitions. This is possible due to the large difference in polarity between the ground and excited state of the probe.[73] At binding sites of significantly different polarity the probe exhibits selective excitation properties and excitation-dependent emission maxima.

16.1.4.4 Combined Fluorescence Quenching Experiments and Paramagnetic Resonance

Data obtained by this technique allow rather detailed conclusions about the location of the solubilizate in the aggregate. Fluorescence quenching rate constants vary with temperature, local microviscosity and quencher concentration.[74–76] Encapsulation of spin probes has been examined in detail by EPR spectroscopy.[77]

16.1.4.5 Cryo-Electron Microscopy

Apart from (bio-)chemical data, direct structural information of vesicles and micelles can be obtained by cryo-electron microscopy. Cryo-electron microscopy as introduced by Jaques Dubochet,[26] has now become a standard technique to gain artifact-free structural information within the natural solvent. In most cases water [78] is used but also other solvents like polar organic solvents[79] and apolar solvents[80] can be employed. Thin layers of solvent with colloidal suspended material can be vitrified by rapid cooling in liquid ethane. By remaining at low temperature inside an electron microscope the solvent will not evaporate in the high vacuum. This allows to gain high resolution structural information.

16.1.4.6 Miscellaneous Techniques

Other techniques that can be used to measure distribution coefficients of a guest molecule between the bulk aqueous phase and a micellar or vesicular aggregate include gel filtration, electromotive force measurements, solubility and vapor pressure measurements, muon spin rotation experiments and headspace gas chromatography.[81] Theoretical models are, of course, also helpful, as, for example, in studies of the enveloping of charged proteins by lipid bilayers.[25]

16.2 Catalysis by Vesicles. Encapsulation of Reactants

Micellar catalysis and inhibition of organic reactions has been studied in great detail.[82] Much less work has been performed on vesicular catalysis and inhibition. The fact that most vesicles are meta-stable aggregates is a reason for this lack of popularity. In order to obtain satisfactory reproducibility of the rate constants, adequate methods for vesicle formation are required and kinetic measurements have to be performed with special care. On the other hand, if the reaction is strongly medium dependent and rate constants can be measured accurately, rather subtle changes in substrate binding location can be nicely revealed. We note here that vesicular catalysis in water can be performed at lower surfactant concentrations than in case of micellar catalysis, which is a significant advantage from the view point of green chemistry.

It will be clear that encapsulation of the reactant(s) is a prerequisite for vesicle-induced rate effects. In case of unimolecular organic reactions, the change in rate constant relative to the rate constant in bulk aqueous solution, is determined by the change in reaction environment going from water to the reactant binding sites in the vesicle. Several studies have suggested that the reaction environment in the vesicular phase is often less polar than that in micelles.[83] Of course, the kinetic effect is a function of the distribution of the reactant over the aqueous and vesicular pseudophases. If the medium effects on the reaction are understood in some detail, the vesicle-induced rate effects provide information about the nature of the reactant encapsulation process.

For bimolecular organic reactions the situation is more complicated. We have now two reactants that bind to the vesicle (sometimes one may stay in the aqueous phase), not necessarily at similar sites in the aggregate. Vesicular catalysis/inhibition now depends on the same environmental factors as described for unimolecular reactions, but also on the binding efficiency, determining the reactant concentrations in the vesicular reaction volume. If reactant binding is sufficiently strong, the local reactant concentrations in the vesicular reaction volume may become greater than those in water, with the concominant higher rate resulting from this 'concentration effect'. Even in the case of a vesicular rate constant smaller than the one in water, catalysis may nevertheless be observed due to the overruling effect of the higher reactant concentrations.

Encapsulation of the reactants in the vesicle can affect rate constants in a number of ways. These include the following: (a) a reduced medium polarity in the Stern region and particularly deeper in the bilayer; (b) a lower water concentration; (c) for charged vesicles: a local electric charge at the surface of the vesicle due to an incomplete counterion binding and a significant ionic strength in the Stern region; (d) rates may respond to the bilayer fluidity which is different below and above the main phase transiton temperature, and finally, the vesicle size[84] may also play a role. We also note that giant vesicles provide interesting microchemical vessels for various types of reactions, including decontamination of mustard-like compounds and polymerization processes.[82]

16.2.1 Unimolecular Decarboxylation of 6-NBIC

The strongly solvent-dependent, unimolecular decarboxylation of sodium 6-nitrobenzisoxazole-3-carboxylate (6-NBIC, Figure 16.4) has been a popular kinetic probe for aqueous media containing surfactant aggregates.

After pioneering work by Kemp and Paul,[85] the main noncovalent interactions which govern the medium effects have been elucidated in some detail by Hilvert *et al.*[86]

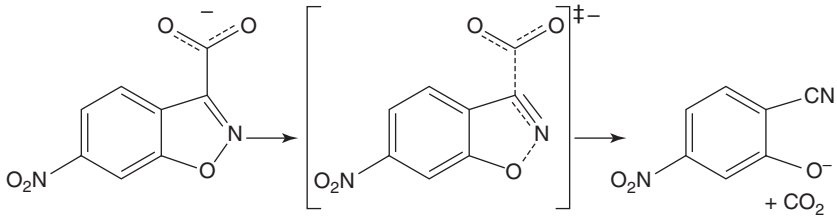

Figure 16.4 *The unimolecular decarboxylation of 6-NBIC*

Hydrogen-bonding stabilization of the carboxylate group as well as ion-pair formation (particularly in apolar, aprotic solvents) retard the reaction by reactant stabilization. Breaking-up these interactions and stabilization of the highly polarizable activated complex (AC) by London dispersion interactions dramatically accelerate the decarboxylation. As anticipated, 6-NBIC binds to positively charged surfaces of surfactant aggregates with the aromatic part of the molecule engaging in hydrophobic interactions with methylene groups of the alkyl tails residing near or at the aggregate's surface.[87,88]

Reaction rates in the presence of cationic surfactant aggregates can be successfully analyzed using the pseudophase model, providing the rate constants for reaction within the aggregate (for vesicles, k_{ves}, taking into account ion exchange processes[89]). Generally, reaction rates increase with decreasing water penetration into the aggregate and with decreasing fluid-like character of the surface of the aggregate:

$$\text{Micelles} < \text{bilayers}\,(T > T_c) < \text{bilayers}\,(T < T_c)^{88}$$

The kinetics of the decarboxylation of 6-NBIC has also been examined in liposome dispersions[83] and in spontaneously formed catanionic vesicles.[90] For equimolar mixtures of *n*-cetyltrimethylammonium bromide (CTAB) and sodium *n*-heptyl sulfate, k_{ves} ($8.29 \times 10^{-4}\,s^{-1}$) is higher than that in pure CTAB ($k_{ves} = 6.76 \times 10^{-4}\,s^{-1}$). Part of this rate increase may be due to the large concentration of NaBr in the solution.

Cationic vesicles, for example those formed from di-*n*-hexadecyldimethylammonium bromide (DHAB) accelerate the decarboxylation by a factor of about 1000 relative to pure water.[91] Dehydration of the carboxylate group at the binding sites is most likely the main factor behind the catalysis. Different isokinetic temperatures (obtained from linear plots of enthalpies *vs*. entropies of activation) have been observed above and below the main phase transition temperature. These excellent isokinetic relationships indicate that the catalytic effects are caused by a single important interaction mechanism.[92]

From the view point of encapsulation, the effects of additives on the nature of vesicular binding sites are of special interest. An important additive is, of course, cholesterol, that plays an important role in life processes. It exerts important effects on the biophysical properties of biomembranes including their fluidity and the formation of microdomains but also on membrane proteins, thereby regulating their functions. Interestingly, there is compelling evidence that phospholipid bilayer systems can form distinct cholesterol-rich and cholesterol-poor domains.[93] Extensive differential scanning microcalorimetric studies on vesicles formed from both synthetic amphiphiles and phospholipids have shown significant effects of cholesterol on the main phase transition temperature, governed by changes in bilayer packing.[94] Compensating enthalpy and entropy effects on melting were found and cholesterol also influences the cooperativity of bilayer melting.

Coming back to the decarboxylation of 6-NBIC, cholesterol was found to reduce k_{ves} for DHAB vesicles by a factor of 3 when at 50 mol % in the bilayer. As a result of its appreciable hydrophobic surface area, it penetrates significantly into the bilayer, thereby decreasing the inter-amphiphile interactions. Under these conditions, the hydration of the interface is increased, and the reactant is stabilized. On the other hand, for trehalose as the additive, the value of k_{ves} is slightly increased. This is in accord with the notion that binding to the bilayer surface leads to replacement of water molecules from the vesicular interface with a concomitant destabilization of bound 6-NBIC.

16.2.2 The Kemp Elimination: Rate-limiting Proton Transfer

A bimolecular reaction, with a substrate that is structurally related, albeit uncharged, and rather similar to the one examined in the previous paragraph, is the Kemp elimination. It involves the rate determining hydroxide-ion induced deprotonation of 5-nitrobenzisoxazole (5-NBI, **1**, Figure 16.5). The high solvent sensitivity of this reaction primarily depends on the reactivity of the OH^- ion.[95] The more weakly the hydroxide ion is solvated, particularly in apolar, aprotic solvents, the stronger the base catalysis. For example, the bimolecular rate constant (k_2) is even 457 times higher in ethanol than in water. In cationic micelles (such as formed from *n*-C_{12}PyrI, DTAB, CTAB, CTACl and OTACl) the organic substrate will bind close to the aqueous interface, participating in both hydrogen bonding and hydrophobic interactions.[96] But what are exactly the properties of this interfacial region as a reaction environment? Indeed, k_2 is also about 400 times higher than in water, in accord with the about similar dielectric constants of ethanol and of the Stern region of the micelles. Plots of k_{obs} *vs*. the surfactant concentration show the typical biphasic pattern for a bimolecular reaction with both substrates partitioning between the bulk aqueous and micellar pseudophase. Hydroxide-ion binding to the micelles depends on the ability of the hydroxide ion to replace the counterion of the cationic micelles.

A kinetic study was also performed in a variety of vesicular solutions (DDAB, DODAB, DODAC; [NaOH] = 2.25 mM, 25 °C).[96] Interestingly, the vesicles possess stronger catalytic reaction environments than the micelles.[97] The rate-determining proton transfer from carbon to the hydroxide ion was accelerated up to 850 fold in di-*n*-dodecyldimethylammonium bromide (DDAB) vesicles. This is evidence that the reaction sides are less aqueous than those in micelles, as anticipated. Application of the pseudophase model afforded the bimolecular rate constants in the vesicles (k_{ves}). For the different vesicles, k_{ves} is significantly higher (*ca*. 12 times for DODAB) than the second-order rate constant in water. This shows that the catalysis is due to both a medium effect and a concentration effect. It was assumed that there was a fast equilibrium for substrate binding to the inner and outer leaflets of the bilayer, in accord with the fact that no two-phase kinetics were found.

Vesicle catalysis followed the order DDAB > DODAC > DODAB, with k_{max}/k_w values of about 850, 550, and 160, respectively. This looked an unexpected result because the rate constants did not respond in the usual way to an increasing chain length of the tails. But it was recognized that the high catalytic efficiency of DDAB with the shortest alkyl chains was the result of vesicles being in the liquid-crystalline state at the reaction

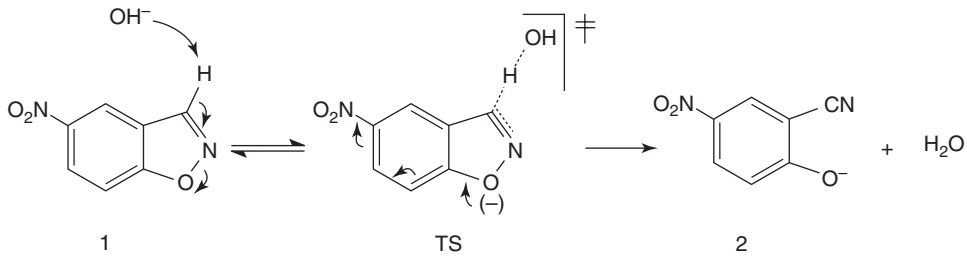

Figure 16.5 *The hydroxide-ion catalyzed Kemp elimination of 5-NBI*

temperature while the other bilayers were in the gel state. A similar effect has been observed before[98].

Addition of cholesterol leads to two counteracting effects on the rate constants. The first is a smaller counterion binding, reducing the rate constants. The other is a rate enhancing effect resulting from the less polar vesicular binding sides. The overall effect depends on the exact reaction conditions.[96]

In a further detailed study the effects of several added long-tailed alcohols and *n*-alkyl pyranosides were examined.[99] The overall results showed that minor structural changes in the additive and concentration of the additive exerted significant changes in the properties of the interfacial region and it was suggested that similar effects can be anticipated for the much more complex biological cell membranes.

Perhaps most interesting was the kinetic response upon addition of *n*-dodecyl-β-glucoside (C_{12}Glu) and *n*-dodecyl-β-maltoside (C_{12}Mal). For C_{12}Glu a rate increase was found, largely due to an increase of k_{ves} since substrate binding (K_s) shows only a minor increase. Using a number of spectroscopic probes, the normalized polarity of the interfacial region was estimated and the data indicated only a minor difference with bulk water.[99] All evidence pointed to a partial dehydration of the interfacial reaction sites induced by C_{12}Glu and a concomitant dehydration of the OH^- ion. Therefore this effect was taken as the origin of the kinetic effects.

Addition of sodium di-*n*-decyl phosphate (DDP) to DODAB vesicles leads to the formation of catanionic vesicles and rather drastic changes in the propertes of the interfacial region. Again the Kemp elimination was employed to probe these changes, in combination with cryo-electron microscopy, DSC, and measurements of the surface charge density and zeta-potentials.[97] Binding site polarties were assessed using Reichardt's E_T-30 probe and pyrene.

Whereas for di-*n*-octadecyldimethylammonium chloride (DODAB) vesicles k_{ves} is about 65 times larger than the water rate constant, addition of DDP decreases the vesicular catalysis. Experiments with the polarity probes indicated that the polarity of the bilayer surface hardly responded to addition of DDP and also the binding constant of 5-NBI was not affected. It was argued that the reduction of the catalytic effect was due to a decrease in counterion binding resulting from addition of DDP.[97] Catalysis could be turned into rate inhibition as was observed for negatively charged vesicles containing 70 mol% DDP in the bilayer. Interestingly, DSC experiments revealed the presence of neutral microdomains ('rafts') in case of 5 and 30 mol % DDP in the bilayer.

16.2.3 Bimolecular Nucleophilic Substitution

Another bimolecular single-step organic reaction which can be used to probe binding sites in vesicular aggregates is the S_N2 reaction of a series of aromatic alkylsulfonates (MNBS; AlkONs, Alk = Me, Et, *n*-Pr, *n*-Hex) with water (Figure 16.6).[100] Now the water is a nucleophile instead of a hydration agent as in the previous probe reactions.

The rates of these hydrolysis reactions (Nu = H_2O) can be compared with nucleophilic substitution by bromide ions (Nu = Br^-). A kinetic study has been made of these reactions in the presence of vesicles formed from synthetic amphiphiles, phospholipids, and mixtures of both types of amphiphiles.[100] Particular attention was paid to the effect of addition of *n*-dodecyl-β-glucoside (C_{12}Glu) as a mimic for glycolipids. Kinetic data were

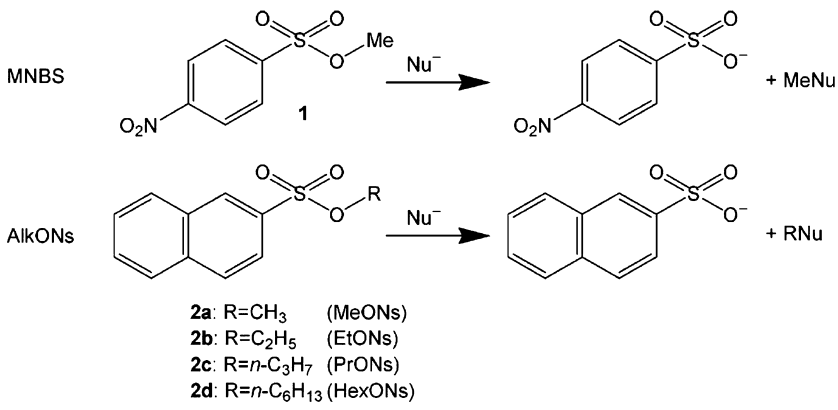

Figure 16.6 *The S_N2 reaction of MNBS and AlkONs with nucleophiles*

analyzed using the pseudo-phase model to afford rate constants for the S_N-2 process in the aqueous phase and in the vesicular pseudo-phase. The experimental data can be reconciled with the reactions occurring at the vesicle–water interphase, with the nature of the binding sites being modified by the presence of C_{12}Glu. It was calculated that for vesicles containing 50 mol% of C_{12}Glu, the vesicular surface is covered for about 34% by C_{12}Glu.

Rate constants for the reactions in the presence of overall positively charged vesicles are about ten times larger than those in the absence of vesicles. The effect was ascribed to an increase in the reactivity of water. In case the water molecules at the vesicular interphase are in part replaced by the glucose groups of C_{12}Glu, the catalytic efficiency of the vesicles decreases significantly.

The bimolecular rate constants for the reaction with bromide ions are smaller at the vesicular interphase, and independent of the presence of C_{12}Glu. These results indicate that this reaction, not involving water as a reactant, is not sensitive towards partial dehydration of the binding sites of the organic substrate.

16.3 Liposomal Encapsulation in Drug Delivery

Since the discovery of liposomal vesicles[9] it has been envisioned that the closed inner compartment of a vesicle can be used to trap potentially harmful substances or to protect unstable compounds from decomposition by shielding them from the outside.[101,102] Despite the high potential of liposomal formulations in drug delivery only a few applications have made it into approved therapeutic drugs. The key to success is the stability of the formulation and the ability to retain the encapsulated compound for a long time once it is loaded into a vesicle. The low solubility and high toxicity and/or unwanted site effects of many anti-cancer drugs make this class of compounds a logic choice for the use of encapsulation. Furthermore the discontinuous vascular blood vessels of tumours are leaky for small 200–1200 nm particles, and liposomes of 100–200 nm readily extravasate at the tumour site.[103]

Efficient liposomal therapeutics also require long circulation times. Normal liposomes are cleared from the blood rapidly by the reticuloendothelial system, but circulation times can be easily increased by giving the liposomes a so-called stealth character.[104] Stealth liposomes are sterically stabilized by lipids with a long polyethylene glycol unit attached to the headgroup, usually phosphatidylethanolamine (PE).[105]

Good encapsulations begin with vesicles in which any compound can be retained for a long time. One way to achieve this is by making the bilayer less permeable for the encapsulated compound. Although a phospholipid membrane is semipermeable, small molecules and monovalent ions can leak in and out, typical at a time span of hours. In the presence of serum, vesicles are usually more leaky than in buffer without serum albumin. Very often cholesterol is added to phospholipids vesicles to make them less permeable as was demonstrated by leakage experiments with carboxyfluorescein (CF) and calcein.[106] Instead of membranes based on (phospho)lipids, the use of block copolymers, with the same basic architecture as lipids but with larger masses, that can form vesicles[107,108] (polymersomes) gains popularity.[109] Another way of making less permeable membranes, involves the use of newly discovered lipids like the laderanes from the anammoxosomes,[110,111] unique organelles in anammox bacteria, and multimethyl branched lipids from the membranes of archeabacteria.[112] Although results on their capabilities are limited at the moment,[113] the synthesis of these lipids became recently available [114–116] allowing large scale experiments on their possible usage as drug delivery capsules.

16.3.1 Encapsulated Drugs

One of the first encapsulated drugs that was reported was liposomal insulin for oral administration instead of the now used method of subcutaneous injection. Insulin, when administrated orally, is broken down in the digestive track, making it useless for sugar uptake from the blood. By encapsulation in bilayer vesicles the idea was that insulin could successfully be administrated orally. The shielding effect of the bilayer to prevent decomposition could facilitate the delivery of intact insulin. The results, however, were inconsistent and an application was never launched. Although the basic idea was good, this method faced the major problems still involved in efficient encapsulation. To retain any encapsulated compound effectively is by making use of the low solubility limit of the compound either by itself or by gelating it with another compound.[117] This was nicely demonstrated for vesicles loaded with the anti-cancer drug doxorubicin or the antibiotic ciprofloxacin.[118] Doxorubicin was actively loaded by a sulphate gradient.[119] Due to the low solubility of the precipitated doxorubicin (Figure 16.7) no leakage was observed even in serum containing media. On the other hand, ciprofloxacin, with a much higher solubility limit leaked out of the vesicles on an hour's time scale.[118] Doxorubicin-loaded vesicles are by now one of the best characterized liposomal drug formulations[120] approved for the treatment of several solid tumours. By encapsulation of the doxorubicin the effective dose could be increased whereas the large site effects, such as the breakdown of cardiac tissue, could be avoided.[121]

Cisplatin is an effective and frequently used anti-cancer drug against a variety of solid tumours. The first trials of encapsulation faced the problem of low drug to lipid ratio due to the limited solubility of cisplatin resulting in low bioavailability.[122]

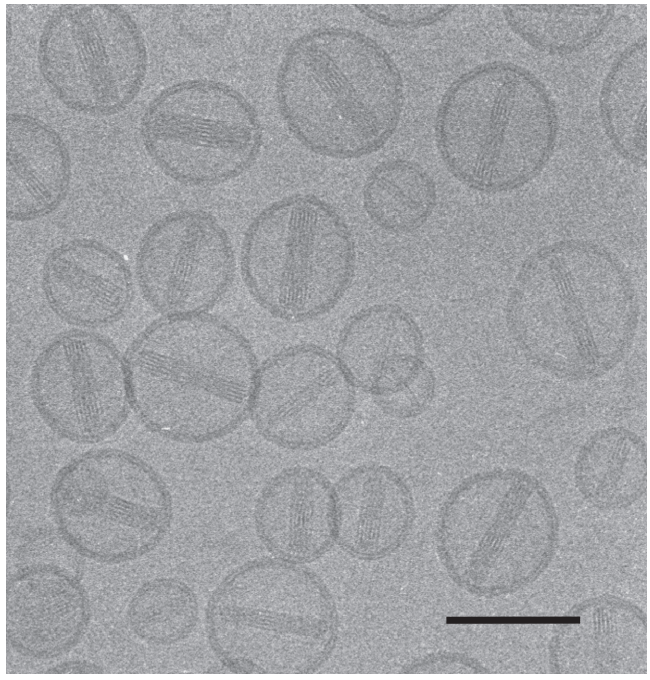

Figure 16.7 *Cryo-electron micrograph of doxorubicin-loaded vesicles. The drug is precipitated into needle-like crystals. Courtesy by Dr. P. M. Frederik, University of Maastricht, The Netherlands. Bar 100 nm*

Effective encapsulation of cisplatin was achieved by multiple freeze thawing steps of neutral and cationic soluble cisplatin in the presence of anionic phospholipids like phosphatidylserine (PS).[123] By freezing the cisplatin is concentrated because the solute is expelled from the ice crystals. When the concentration limit is reached the neutral cisplatin, with the lowest solubility limit, aggregates followed by aggregation of positively-charged cisplatin when the solution further concentrates during the freezing. The negatively-charged PS collapses on the small particles resulting in small aggregates covered by lipids.[124] In this way very high cisplatin to lipid ratios were achieved.

Today more than ten liposomal drug formulations have been approved for clinical use[125,126] and the number is growing. Ideally a liposomal encapsulated drug exhibits hardly any drug release in the blood plasma, but once at the designated site all its content should be released.

To trust only on mechanical instability by accumulation on a desired spot for the release of pay-load from vesicles is an imperfection of the system. By the incorporation of channel proteins in the bilayer of vesicles that can open or close upon a variety of signals[127] smart release of encapsulated substances is brought closer. Especially the modified mechano-sensitive channel protein MscL[128,129] from Lactococcus lactis can be remotely controlled. The MscL can be modified to be responsive to light or pH[130–132] for the release of encapsulated compounds.

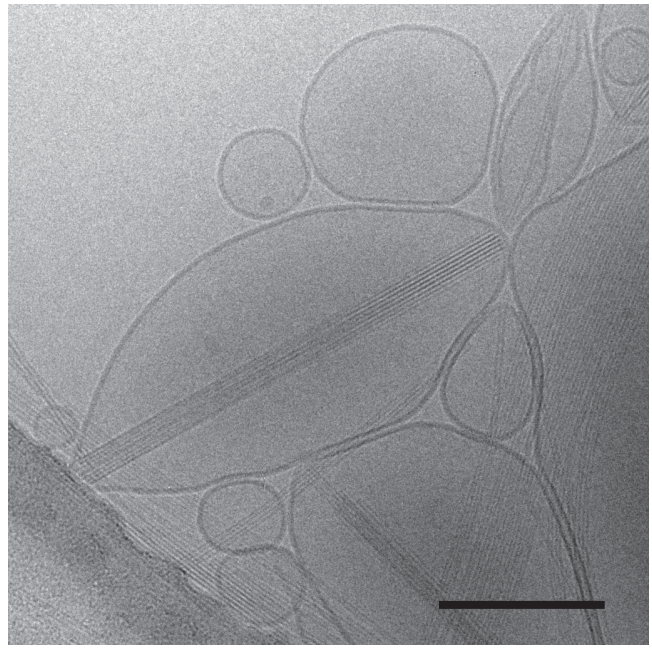

Figure 16.8 *Gellosomes, growth of gel-fibers directly in the aqueous compartement of a phospholipid vesicle. Reproduced with permission from Wiley-VCH Verlag GmbH & Co. KGaA*

An alternative for encapsulation in vesicles is the use of low molecular weight hydrogelators. Gels can retain solvents and solutes in the space between a dense network of intertwined fibres. Similar to encapsulated material by vesicles, gels can retain compounds. Aqueous hydrogelators based on cyclohexyl-tris-amino acid can self-assemble in one direction through the establishment of H-bonds, leading to the formation of a fibre network and consequently macroscopic gels.[133] The formation of these gel networks was found to be independent from the self-assembly of phospholipids into vesicles which is driven by hydrophobic interaction. By encapsulating a gel network into liposomes (Figure 16.8) the advantages of both liposomes and gel networks can be combined in a single system.[134]

Despite the high expectations only a handful of applications of liposome- encapsulated drugs is available. Nevertheless the high potency of liposomes is still considered as very useful in this field and research is progressing.

16.4 Vesicle–Nucleic Acid Interactions: Gene Transfer Using Lipoplexes

Genetic modification of living cells can be accomplished by delivering exogenous genetic material into the cell thereby replacing a missing or deficient gene with the accompanying therapeutic effects and improved cell biological functions. Medical applications in the

clinique have already been successful using viral vectors for DNA trafficking[135] During the past decades, extensive research has been performed on the synthesis and testing of *non-viral* vectors for delivering nucleic acids into living cells, both *in vitro* and *in vivo*. Examples include DOTMA (*N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride), DOTAB (*n*-dodecyl-trimethylammonium bromide), SAINT-2 (*N*-methyl-4-(dioleoyl)methylpyridinium chloride and sugar-based gemini surfactants. This work makes use of some of the most sophisticated applications of bilayer encapsulation. The vectors include cationic, bilayer-forming amphiphiles,[136–138] which are viewed as useful alternatives for virus-based delivery agents which may be associated with mutational and immunological hazards. These carriers are often used in combination with specific phospholipids. The transfection efficiencies of different commercially available cationic lipid-based transfection kits have been compared.[139] A number of biodegradable pyridinium amphiphiles have also been synthesized and exhibited remarkably high transfection efficiencies.[140]

The literature on gene transfection is enormous and the results have been reviewed.[137,141,142] We will focus here exclusively on nucleic acid encapsulation by vesicles and discuss some of the factors governing the interactions. The resulting lipoplexes may contain entire genes, (antisense) oligonucleotides[143–145] or RNA and small interference RNA (siRNA) and are in most cases obtained by adding the nucleic acid to preformed vesicles.

16.4.1 Lipoplex Formation

Lipoplex formation is very efficient (occurring on an ms time scale) with *cationic* amphiphiles, indicating that the binding is primarily electrostatically driven. The ratio amphiphile/DNA is such that all negative charges of the DNA are involved in binding to positively charged headgroups of the amphiphiles, thereby leading to a concomitant condensation of DNA to a compact toroidal structure. There should be sufficient additional amphiphile to provide the lipoplex with an overall positive charge. These lipoplexes can then favorably interact with the negatively charged cell surface, for example with anionic proteoglycans.

Liposomes formed from natural phospholipids are either zwitterionic or carry a net negative charge. Using them as gene carriers, nucleic-acid binding must now rely mainly on entrapment into the aqueous pool of the liposomes.

There is compelling evidence that the efficiency of the delivery vehicle depends strongly on the ability of the lipoplex to interact with the cell surface, necessary for induction of transfer of the gene into the cellular interior. As will be discussed later, the cationic amphiphiles should possess the necessary properties for affecting transport *across* the membrane and they should allow destabilization of the endosomal membrane and subsequent release of DNA into the cytosol for trafficking to the nuclear membrane. The latter step is a key factor in gene transfection. A variety of issues play a role here, including a favorable chemical constitution of the amphiphilic carrier, environmental factors, and intermingling of the vesicular phase of the lipoplex with selected phospholipids that can provide endosomal membrane destabilization and a concomitant release of the bound DNA. *Endocytosis* is often considered to be the major entry pathway for this process. It has been shown that the complex internalization process can occur via the cholesterol-dependent clathrin-mediated pathway of endocytosis.[146,147] Evidence includes

the observation that transfection is greatly inhibited when plasma membrane cholesterol is depleted with methyl-β-cyclodextrin whereas cell-association remains unchanged.

Then, when delivered into the cytosol, the gene has to find its way to the cell nucleus, the site where transcription and replication will occur. A detailed recent study showed the crucial importance of nuclear transcription efficiencies.[148] In fact there are many individual steps that make up the gene transfection and mechanistic studies are performed to identify these steps and to find ways to facilitate these steps.

16.4.2 Lipoplex Structure

Lipoplexes can have several morphologies each possessing different transfection efficiencies.[142] The most frequently encountered structural phase is lamellar (L^c_α) in which the DNA is sandwiched between the lamellae (Figure 16.9), as indicated by high resolution cryo-electron microscopy and SAXS measurements[149] as well as by X-ray diffraction.[150] Their average size is about 200 nm, allowing a few of these lipoplexes to fit within an endosomal compartment. Mixing the cationic carrier with the phospholipid DOPE (mole fraction >0.4) leads to formation of a highly curved mesomorphic morphology, in this case an inverted hexagonal structure (H^c_{II}) containing DNA rods coated with a monolayer of the amphiphile arranged on a hexagonal lattice. This phase is the favourable one for inducing efficient release of the DNA cargo from an (early) endosomal compartment in vitro. The hydrophobic outer-surface of inverted hexagonal lipoplexes make them not suitable for *in vivo* use. It has also been established that the H^c_{II} phase plays an important role in destabilizing the endosomal membrane, thereby inducing efficient translocation of DNA across the endosomal membrane into the cytosol.[151]

The effect of the helper lipid on the lipoplex morphology depends on control of the spontaneous radius of curvature of the bilayers and is dependent on its mole fraction (mf) in the bilayer. For example, for DOTAB/DOPE cocktails, a pure lamellar phase is formed when mf(DOPE) is <0.41. A pure H^c_{II} phase is formed when mf(DOPE) is >0.75,

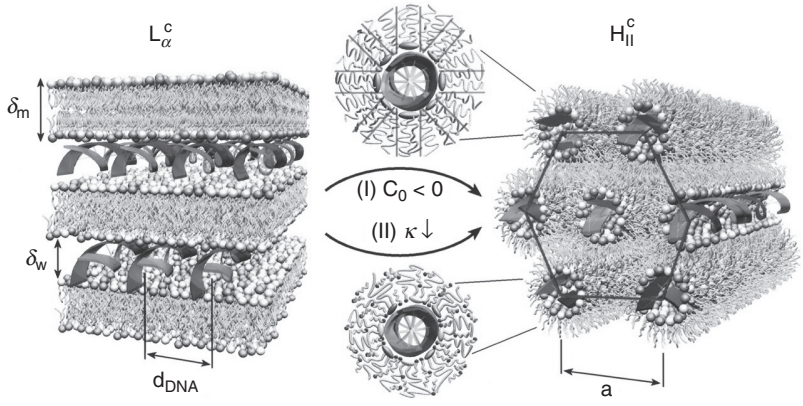

Figure 16.9 *The L^c_α and H^c_{II} morphologies of lipoplexes formed from DNA and cationic bilayer-forming amphiphilles. Reprinted with permission from AAAS*

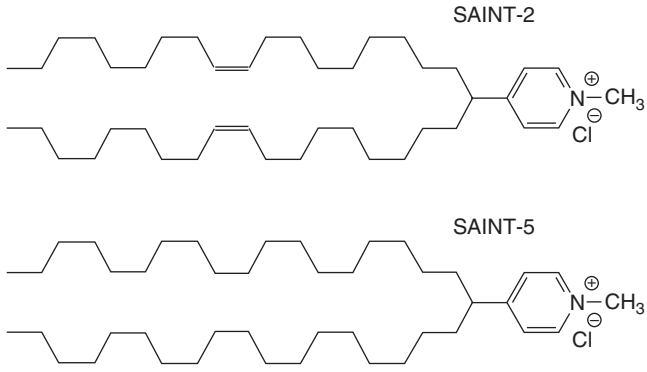

Figure 16.10 *Molecular structures of SAINT-2 and SAINT-5*

whereas both morphologies coexist for 0.41 < mf(DOPE) < 0.75. Increasing mole fractions of DOPE lead to gradual dehydation of the DNA bases. Transformation of DOTAP/DOPE systems into the H_{II}^c phase was evidenced by small angle X-ray scattering (SAXS) and optical microscopy.[152] For SAINT-2/DOPE cocktails a similar phase change, but now at high salt concentrations, was shown by NMR spectroscopy and SAXS measurements.[153,145,154]

The transition from L_α^c to H_{II}^c can be rationalized by considering the packing parameter P (section 16.1.1). For bilayer forming carrier molecules, P is between 0.5 and 1.0, often close to 1.0; by contrast the phospholipid DOPE is cone-shaped and P is > 1, leading to a preferred formation of an inverted hexagonal phase.

There is evidence that a transient spaghetti-like morphology can be formed between the L_α^c and H_{II}^c phases, acting as a precursor to the H_{II}^c morphology.[155]

We like to emphasize here, that the efficiency of a helper lipid like DOPE is, however, influenced by the packing of the alkyl tails in the membrane formed by the cationic carrier amphiphile. This was demonstrated[156] by a comparative study of two structurally related bilayer-forming surfactants, SAINT-2 (with two C18:1 tails) and SAINT-5 (with two C18:0 tails) (Figure 16.10). Both carriers display transfection activity, but DOPE exerts a positive effect on SAINT-2-mediated transfection, but has a negligible effect on transfection mediated by SAINT-5. Interestingly, DOPE effectively enhances DNA dissociation from the lipoplexes formed from both carriers. Most likely, membrane stiffness plays an important role here. Since the bilayer composed of SAINT-5 is more rigid than that formed from SAINT-2, because of the absence of unsaturation in the tails, the plasmid DNA becomes less effectively condensed, and the lipoplex is structurally deformed. This has no effect on cellular uptake but reduces the efficiency of translocation of the plasmid across the membranes of the endosome and/or of the cell nucleus.

In contrast with supercoiled DNA, steric factors most likely prevent translocation of the uncondensed DNA from the endosome into the cytosol. Consistent with this interpretation, the much smaller oligonucleotides are effectively translocated into cells by lipoplexes formed from both SAINTs. In case the plasmid is stabilized by condensation with poly-L-lysine, the transfection by SAINT-5/DOPE is greatly improved. The observed phenomena illustrate that the structural shape of the plasmid is a substantial factor in

transfection processes. This factor has been considered previously but without reaching consensus.[157]

A third morphology that has recently been identified is an intercalated hexagonal structure in which three honeycombs of amphiphile micelles cover the DNA rods, thereby forming a normal, hexagonal lattice (H_I^c). This was found for the two sugar-based gemini surfactants GS1 and GS2 (Figure 16.11), for which the lipoplex forms an L_α^c phase at physiological pH values as indicated by cryo-EM and SAXS[158] measurements. The phase transition after internalization of the lipoplex into the endosomes with a mildly acidic pH was examined using the solvatochromic fluorescent probe Nile Red.[73] In the resulting H_I^c phase the polar headgroups of the geminis are exposed on the outside in contact with water.[159] This in contrast to the earlier proposed H_{II}^c phase for the lipoplexes.[158] As a consequence, these particles exhibit an unusually high colloidal stability (as shown by turbidity measurements) facilitating their application in *in vivo* gene delivery experiments.[160] In the H_{II}^c phase the polar headgroups of the amphiphile participate in

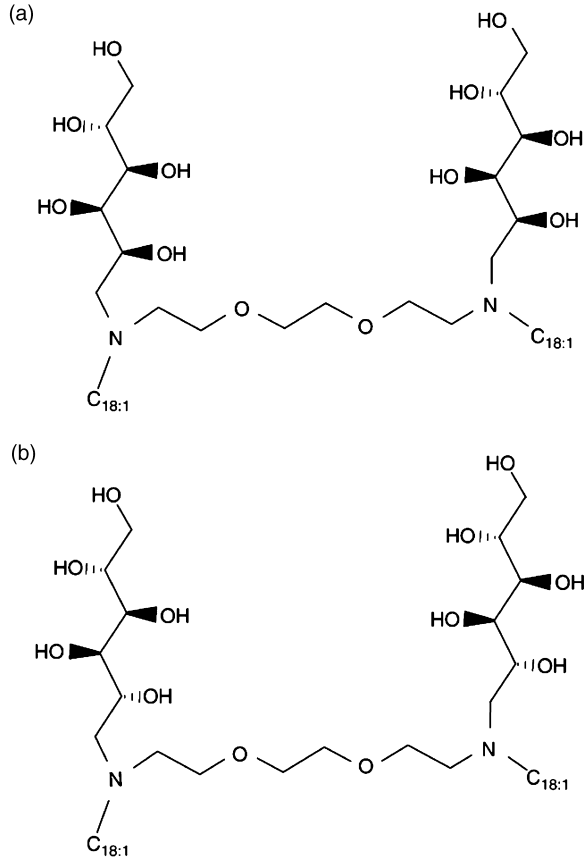

Figure 16.11 *Molecular structures of the reduced sugar-based gemini surfactants GS1 (a) and GS2 (b). Note the different stereochemistry of the sugar moieties*

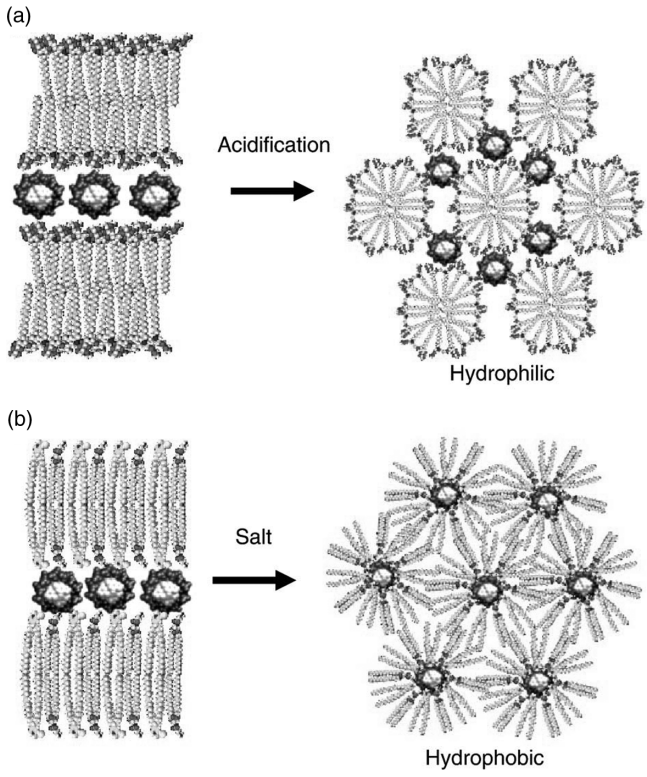

Figure 16.12 *The H_I (GS1,2) (a) and H_{II} (SAINT-2/DOPE with added salt) phases (b) lipoplex morphologies. Reprinted with permission of Elsevier. Copyright 2006*

electrostatic interactions with the DNA and the hydrophobic tails are exposed on the outside where they can easily induce aggregation behaviour and capture in the lung endothelium capillaries. This problem has been tackled by coating the lipoplexes with PEG-lipids that enhance the blood circulation times.

In Figure 16.12 the morphology of the H_I^c phase is compared with that of the H_{II}^c phase formed from SAINT-2 and DOPE. A similar H_I^c phase has previously been reported for lipoplexes composed of the single-tailed, micelle-forming surfactant CTAB.[161] In this phase the amphiphile monomers are dynamic and able to move in and out of the micelles. These properties allow translocation of endosomal lipids into the lipoplex thereby stimulating release of DNA into the cytosol.

Oligonucleotides (ODNs) can function as effective gene-specific regulators and have considerable therapeutic potential. Although small quantities of ODNs can be endocytosized via adsorption, it is necessary to prevent their sequestering in endocytic compartments. It has been shown[144] that SAINT-2 can act as an effective carrier for specific antisense ODNs to target mRNA. No problems with cytotoxicity were found. Using Chinese hamster ovary cells, the protein levels of the receptor for the neuropeptide

corticotropin- releasing factor were assessed. ODN release did not depend on the size of the lipoplex and the presence of serum. However, in case serum proteins are incorporated into the lipoplex, the lipoplex membrane is stabilized, hampering ODN release.

For *in vivo* applications, the circulation time of the ODN-carrier complex has to be increased. This was accomplished for the same cell type by incorporating complexes of PEG with either phosphatidylethanolamine (PE) or ceramide.[145] The data suggested that cytosolic release of the ODNs from the endosomal compartment was inhibited by the PEG-lipid through stabilization of the lamellar phase of the lipoplexes. For a more detailed discussion of this important field, the interested reader is referred to recent reviews.[162,143]

Rather surprisingly, it was found possible to form a lipoplex from DNA and a zwitterionic multilamellar phase at high lipid/DNA weight ratios.[163] The impetus for these studies was the lower cytotoxicity of neutral (and also negatively charged) liposomes. Encapsulation of DNA by soya bean diacylphosphatidylcholine (PC) was carried out by mixing the lipid (containing a small amount of a helper surfactant) and short DNA fragments (*ca*. 150 base pairs) in excess water and subsequent freeze-drying. The resulting dry powder was then hydrated with deionized water. SAXS measurements on these systems were in accord with a multilamellar structure with intercalated monolayer DNA between the neutral lipid layers, despite the absence of electrostatic binding interactions. The absence of significant DNA-lipid interactions was experimentally verified and as a consequence the DNA molecules possess more motional freedom than in the conventional L^c_α complexes formed from cationic carrier systems.

Another remarkable lipoplex morphology was proposed in experiments in which short DNA fragments (either pure or marked with a fluorescent dye) were locally injected, using a micropipette, into a part of the membrane of a giant unilamellar vesicle (GUV, diameter *ca*. 100 μm). These vesicles were formed from phosphatidylcholines and upto 33 mol% of a cationic sphingosine by electroformation.[164] Membrane topology was observed in phase contrast, DNA distributions by fluorescence spectroscopy. Local DNA-lipid interactions in the membrane induced endocytosis, which needed a minimum concentration of D-erythrosphingosine. At lower concentrations only lateral adhesions between neighboring vesicles were found upon local addition of DNA. The size and shapes of the endosomes were dependent on the kind of DNA and the initial GUV membrane tension.

Although the evidence was not fully compelling, it was suggested that DNA-lipid interactions involved DNA encapsulation within a cylindrical inverted micelle, included in the lipid membrane (Figure 16.13).

Gene expression was verified in cell-sized, giant vesicles formed from DOPC/DOPG (10 : 1). In individual vesicles expression was found of red-shifted green fluorescent protein (rsGFP) using fluorescence spectroscopy.[165] Particularly in the early stage of the reaction, expression inside the vesicle was significantly higher than that in bulk aqueous solution. Interestingly, the rsGFP synthesized in the vesicles is protected from attack by proteinase K that was added to the external aqueous medium.

A lot of work has been done to find out how lipoplexes and lipoplex-cell interactions respond to the presence of serum. Many relevant references are cited in a paper published in 2002[166] that reports how lipoplex stability and processing are affected by serum. The cationic surfactant carrier was SAINT-2 using DOPE as the helper lipid. Previous studies had already shown that transfection efficiencies are reduced in the presence of serum, in

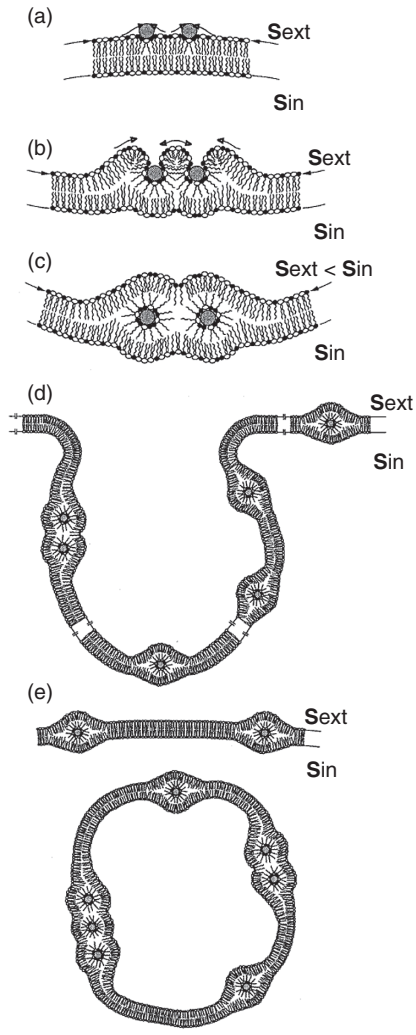

Figure 16.13 *Suggested mechanism for endosome formation of short DNA and GUV membranes. (a) DNA adsorption to the planar GUV membrane (dashed circles represents transverse sections of the DNA molecules). (b) Lateral diffusion and increase of the Sph+ concentration, decoupling of both monolayers, and external monolayer 'rolling' up on the DNA molecules. (c) Topological transformation of the external lipid monolayers and encapsulation of DNA within a cylindrical inverted micellar structure. Membrane asymmetry is created ($S_{ext} < S_m$). (d) Membrane invaginates at a scale of a few micrometers. (e) Formation of the endosome. Reprinted with kind permission of Springer Science+Business Media*

accord with the results for SAINT-2 lipoplexes. In the absence of serum, clustered lipoplexes ($^{-}$FBS lipoplexes, FBS is fetal bovine serum) were observed. Clustering did *not* occur in the presence of serum ($^{+}$FBS lipoplexes) or when serum was present during lipoplex formation (FBS lipoplexes). Interestingly, the topology of DNA in FBS lipoplexes changes from a supercoiled conformation (as also in $^{-}$FBS lipoplexes) to a predominantly open-circular conformation. This leads to faster digestion by DNase. Most significant was the observation that internalization of $^{-}$FBS and $^{+}$FBS lipoplexes is about three times slower than that of FBS lipoplexes although their transfection efficiencies are about five times higher. The data indicate that smaller serum protein-penetrated particles cannot fast enough release their DNA from pre-lysosomal endocytic compartments and are delivered to lysosomes where they are prone to degradation pathways. Size and morphology of the lipoplex govern their ability to interact with and perturb cell membranes, processes that are needed for release of the gene. Serum regulates these processes in an amphiphile-dependent manner through complex 'penetration' and modulation of the DNA conformation.

16.4.3 Future Prospects

It will be clear that DNA encapsulation by vesicles is the starting point for gene transfection. In a complex follow-up, DNA is finally transported to the cell nucleus. The variety of factors determining transfection efficiencies, including lipoplex size and zeta potential, incubation time, cytotoxicity and lipoplex morphology, both *in vitro* and *in vivo*, are still under active investigation. The final goal is a successful and safe application in the clinique. Our attention here was only focused on lipoplex formation, their structure and properties. The results obtained so far overwhelmingly show the complex features of DNA encapsulation by the cationic amphiphilic carrier systems. Insight into these issues as embedded in a realistic mechanistic picture of the whole transfection process might ultimately give the desired success.

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